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13. ABSTRACT ***(Maximum 200 Words)***

Although most of the tumors associated with neurofibromatosis type 1 (NF1) are benign in nature, malignant transformation of a subset of NF1 tumors is a serious complication, often leading to the death of the patient. This is true for NF1-associated juvenile myelomonocytic leukemia (JMML), known to progress into acute myeloid leukemia (AML). Previously,we have shown that loss of *Nf1* in the hematopoetic lineage results in JMML. The goal of this grant is to identify the genetic events which lead to the transformation and leukemic progression of NF1-associated JMML using a mouse model. This model system takes advantage of transgenic mice that harbor one mutant allele of the *Nf1* gene, but require further mutations for transformation. We have backcrossed this mutant *Nf1* allele for three generations to a strain of mouse that expresses a murine leukemia virus (MuLV). In this system, the MuLV acts as a mutagen to activate cooperating cellular proto-oncogenes or inactivate tumor suppressor genes, resulting in accelerated tumor development. So far, by cloning sites of somatic MuLV integration from tumor DNAs obtained from these backcrossed mice, we have identified three loci that appear to cooperate with the loss of *Nf1* to cause to AML.

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Introduction

Children with neurofibromatosis type 1 (NF1) have a markedly increased risk for juvenile myelomonocytic leukemia (JMML). JMML has a poor prognosis, with either progression to acute myeloid leukemia or death from bleeding or infection. Using a mouse model of NF1, we demonstrated that the lack of the *Nf1* gene in hematopoietic cells is sufficient to cause chronic myeloid leukemia, but not acute myeloid leukemia, indicating that additional genetic events are responsible for the progression from chronic to acute disease. The goal of this proposal is to identify and isolate genes involved in this leukemic progression. Toward this end, we are using a mouse model system which takes advantage of mice that harbor one mutant allele of the *Nf1* gene but require further mutation for transformation to neoplasia. The general strategy is to breed the mutant *Nf1* allele onto a strain of mouse that expresses murine leukemia virus (MuLV) and exhibits a high incidence of acute myeloid leukemia. In this system, the MuLV acts as a somatic mutagen to activate cooperating cellular proto-oncogenes or inactivate tumor suppressor genes, resulting in accelerated tumor development. Since MuLVs activate proto-oncogenes by integrating nearby or inactivate tumor suppressor genes by integrating within the gene, the affected genes can thus be identified and cloned using these somatically acquired viruses as signposts. The most promising loci are the ones that have sustained proviral insertion in tumors of multiple mice. The identification of a so-called "common site of viral integration" strongly indicates that the region harbors a gene, that when mutated by the virus, is directly involved in the development of myeloid leukemia. To date, we have identified three common sites of viral integration and are in the process of identifying and/or characterizing the gene affected by each site.

Technical Objective 1: Identify regions of the genome that cooperate with loss of *Nf1* in myeloid leukemia to cause tumor progression. Our objective is to produce a panel of 100 tumors derived from heterozygous N3 generation BXH-2 *Nf1*^{Fcr}/+ mice. This panel will then be used to identify new common sites of viral integration. This objective is divided into six tasks.

Progress on Task 1: *Produce F1, then N2, followed by N3 BXH-2 Nf1*^{Fcr}*/+ mice.*
Our initial strategy was to complete production of each generation before going onto the next. However, in practice, this strategy has not worked, mainly because the parental BXH-2 strain to which we are backcrossing has had small litters. Therefore, we staggered the breeding to keep in line with the BXH-2 production.

Progress on Task 2: *Age N3 generation BXH-2 Nf1*^{Fcr}*/+ mice.*
At the time of the 1999 annual report, we had 33 animals in aging and were confident that the project would satisfy the goal of isolating lymph node tumors from approximately 100 independent animals by the end of the budget period. However, in February 2000, all of the 33 mice in aging died due to a bacterial infection that spread through the mouse room. Therefore, we requested and were awarded a one year no-cost extension. We have reimported our mouse stocks and during the coming year, we will repeat the required three generations of breeding and then age the resulting N3 mice. This will ensure that the we will eventually reach the 100 tumor goal.

Progress on Task 3: *Collect moribund animals. Process tumor sample. Assess the status of the wild-type Nf1 allele; determine the number of somatically acquired viral integrations; phenotypic analysis.*
To date, we have collected tumors from 66 N3 BXH-2 *Nf1*^{Fcr}/+ tumors. Each of these tumors have been characterized for loss of the remaining normal *Nf1* allele and the number of somatically acquired viral integrations. We have determined that 59 of these tumors contain either LOH for *Nf1* or a viral integration at *Evi-2*. Therefore, by backcrossing the *Nf1*^{Fcr} mutation onto the BXH-2 background, we have increased the incidence of tumors involving the *Nf1*-pathway from the 10-15% observed in the parental BXH-2 strain to an incidence of 89%. Finally, we determined the number of somatic viral integrations for each tumor. The tumors have a mean number of 3 such viral integrations.

Progress on Task 4: *Clone somatically acquired proviruses from tumor samples which harbor defects in the wild-type Nf1 allele and have only one or two somatic proviral insertions. Prepare genomic flanking probes to screen tumor panel.*
This year, we cloned 4 new sites of viral integration. After the identification of non-repetitive flanking probes, we screened the tumor panel prepared in Task 5 and determined that one recognized a common site of viral integration (*Epi3*, see below).

Progress on Task 5: *Prepare Southern blot containing the panel of tumor DNA samples which harbor defects in the wild-type Nf1 allele. Screen with probes to identify common sites of viral integration.*
The Southern blots have been prepared using all the available tumor samples. Using these Southern blots, we have been able to identify three common sites of viral integration:

Epi1, affected in 48% of the tumors. For progress report, see Technical Objective 2

Epi2 affected in 3% of the tumors. For progress report, see Technical Objective 1, Task 6

Epi3 affected in 3% of the tumors. No more information as of yet.

Progress on Task 6: *Use probes that recognize common sites of viral insertion to isolate cosmid DNA. Begin studies to identify and characterize affected gene.*
The lone somatic viral integration site was cloned from tumor #355. A genomic fragment flanking the site of viral integration was used to screen the tumor panel and rearrangements were detected in several independent tumors. Initially, we named this common site *Epi1* (Ecotropic proviral integration site 1). The genomic flanking probe was then used to genetically map the common site of viral integration to chromosome 10, cosegregating with the c-*myb* gene. Using a c-*myb* cDNA probe, we determined that both the common site and the c-*myb* gene were contained on a BAC clone, indicating that the two loci were physically linked. In the past year, we have isolated multiple probes downstream of the c-*myb* gene and identified 16 additional tumors with viral integrations, bringing the total to 32 tumors (48% of the panel). Table 1 lists tumors that identified as having a viral integration in or near *c-myb*. Currently, we are isolating more probes from the BAC clone to determine if we can detect any additional rearrangements in other tumors in the panel in the vicinity of *c-myb*.

Most previously reported viral integrations proven to affect c-*myb* have occurred within the c-*myb* gene and result in overexpression of a truncated oncogenic product (Wolff, 1996). However, our working hypothesis is that viral integrations at *Epi1* do affect the c-*myb* gene. To determine how 3' viral insertions affect c-*myb* expression, we examined the orientation of the virus in 12 of the tumors in which we knew contained full length virus (and hence were able to orient). We found that in all 12 cases, the virus had integrated in the same transcriptional orientation as the c-*myb* gene. These data are consistent with gene activation occuring by an enhancer insertion mechanism. With this in mind, we assayed the level of c-*myb* expression in the tumors using a Northern blot containing total RNA isolated from several of tumors with and one without a viral integration near c-*myb*. We found that c-*myb* expression levels do not appear to be increased in tumors containing a viral insertion near the c-*myb* locus relative to one lacking such a viral insertion (data not shown). However, it is not clear if a tumor lacking a 3' c-*myb* insertion represents an appropriate negative control, because it is still a tumor

Table 1. Tumors with viral integrations near the c-*myb* locus

Animal #	LOH	*Evi-2*	# of viruses	c-*myb* insertio
7	+	-	4	within
8	+	-	2	within
10	-	-	3	3'
11	-	-	1	3'
13	+	-	3	3'
14	-	+	2	3'
33	+	-	3	3'
35	+	-	5	3'
46	-	+	2	3'
56	+	-	2	3'
84	+	-	2	3'
103	+	-	2	within + 3'
355	+	-	1	3'
356	-	+	3	3'
358	+	-	2	3'
368	+	-	5	3'
371	+	-	1	3'
419	+	-	2	3'
468	+	-	2	3'
522	+	-	5	3'
570	+	-	2	3'
603	-	-	1	3'
639	-	+	2	3'
647	+	+	2	3'
660	-	+	3	3'
662	+	-	3	3'
665	-	+	3	3'
671	+	-	2	3'
675	-	-	2	3'
712	-	-	3	3'
725	-	+	1	3'
1858	-	+	1	3'

and it may have upregulated c-*myb* in another manner. Certainly, normal bone marrow has little to no c-*myb* expression (data not shown), but bone marrow may not represent a reasonable negative control either. Therefore, we hypothesize that the presence of the viral *cis*-regulatory elements downstream of c-*myb* do affect c-*myb* expression, but not by upregulating transcription. Rather, we speculate that the virus serves to prevent transcriptional downregulation of c-*myb*, a process known to be required for terminal differentiation of the myeloid (reviewed in (Oh, 1999)). We anticipate publication of these results in the coming year.

Finally, 94% of the tumors harboring viral integrations near c-*myb* also exhibit either LOH for *Nf1* or have an *Evi-2* integration (Table 1). Since it is possible that the remaining 6% contain a different inactivating mutation in the large *Nf1* gene, our data suggest that loss of *Nf1* can cooperate with c-*myb* deregulation. However, a key question is if these two events are sufficient to cause progression to acute leukemia. Certainly we have identified 5 tumors with LOH or *Evi-2* that contain only one additional viral integration at the c-*myb* locus (Table 1, tumors 14, 46, 355, 371 and 639), but these tumors may harbor other somatic mutations that have escaped our detection. Therefore, to properly determine whether these two genetic events are sufficient to cause progression to acute leukemia, in the future, we will need to recapitulate the loss of *Nf1* together with c-*myb* deregulation using a more genetically-defined system.

Technical Objective 2: Characterize a locus containing a gene involved in tumor progression. Our objective is to identify the gene that is affected by the *Epi2* common site of viral integration. We have divided this objective into 4 tasks (note: we combined the progress report for Tasks 1 and 2).

Progress on Tasks 1 and 2: *Exon trap cosmid isolated with flanking probe. Identify gene.*
As the exon trap approach did not work, we sought alternate means to identify the affected gene. In collaboration with the Copeland and Jenkins laboratory, *Epi2* was mapped to a region of mouse chromosome 17 syntenic to human chromosome 6p21 and cosegregated with two known genes. To determine if either genetically linked gene was physically linked to our common site, we screened the Research Genetics BAC library with the common site probe and identified two BAC clones. We found that neither gene was on the BAC clones and thus eliminated them as candidate genes. To identify to the gene affected by these viral integrations, we sequenced 2.7 kb of the genomic DNA adjacent to the site of viral insertion. We found this flanking DNA to be homologous to a 6p21 human genomic clone deposited in Genbank (accession number AC006049). Through comparison of GRAIL-predicted exons and the regions of highest homology between our genomic sequence and the human

genomic clone, we identified two potential exons, both of which contained an open reading frame. We used the predicted open reading frame to search Genbank and identified three nearly identical, but independently isolated human cDNA clones: PTD017 (AF100761) isolated from a pituitary tumor, HSPC183 (AF151017) isolated from CD34+ stem cells, and DKFZp564H0223 (AL050361) isolated from an unknown source. We have compared these cDNA clones to the human genomic sequence (AC006049) and deduced that the human gene contains at least seven exons.

Progress on Task 3: *Isolate cDNA clones. Determine the genomic structure and normal expression pattern of the gene. Determine orientation of the gene relative to the viral integrations. Establish if an virus activates or eliminates gene expression.*

Recent RT-PCR analysis has shown that expression of the two exons was detected in all tissues examined (Fig. 1), including a the tumor with a viral integration at this site (419) and one tumor without (371).

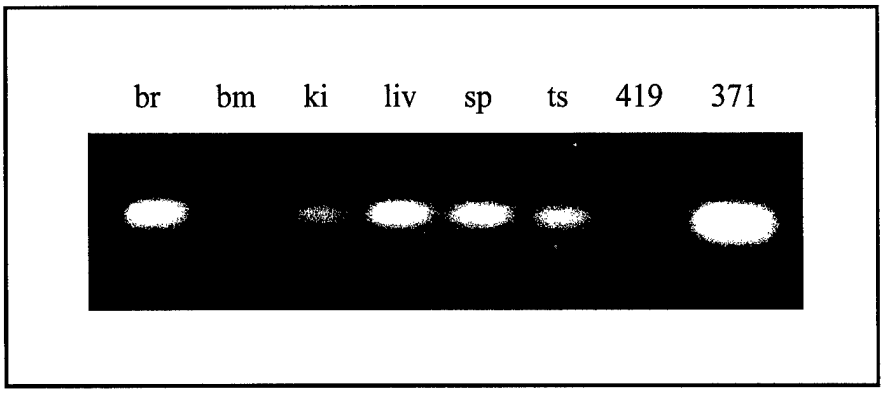

Figure 1. RT-PCR expression analysis of *Epi2* in several murine tissues. RNA derived from brain (br), bone marrow (bm), kidney (ki), liver (liv), spleen (sp), testes (ts), tumor 419 (with viral integration) and tumor 371 (without viral integration) was each subjected to RT-PCR using primers in the two putative exons. The minus RT PCR control was blank for all samples (not shown)

Due to the recent information derived from the sequence and expression analysis as described above, we now have obtained evidence that we have identified the murine homolog of a previously cloned (but unpublished) human gene. Provided the gene structure is conserved in mouse, our sequencing data indicates that the exons detected by RT-PCR correspond to exons 3 and 4, and that both proviruses

have integrated in the second intron and in the same transcriptional orientation as the gene (Fig. 2*a*). Therefore, it would appear that the proviruses have mutated the "*Epi2*" gene by one of two protein truncation mechanisms: either via premature transcriptional termination, resulting in a 3' protein truncation (Fig. 2*b*), or by promoter insertion, resulting in a 5' protein truncation (Fig. 2*c*). To determine which of these two possible mechanisms are at work, we will first characterize the murine gene and then look for a fusion transcript between *Epi2* and viral elements.

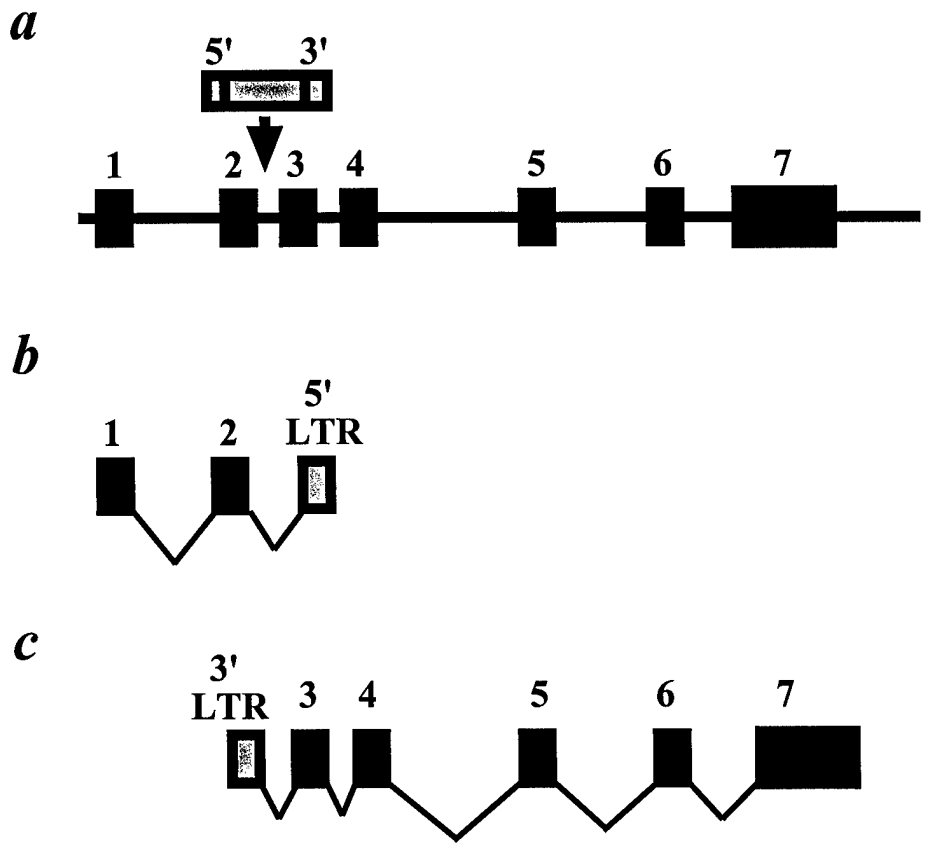

Figure 2. Putative genomic arrangement of the *Epi2* gene and possible consequences of viral integration. (*a*) The structure based on the human gene and the approximate location of the two independent viral integrations. (*b*) 3' protein truncation mechanism caused by transcription initiation at the normal promoter followed by premature transcriptional termination in the 5' viral long terminal repeat (LTR). (*c*) 5' protein truncation mechanism caused by transcription initiation from the 3' LTR (or possibly the 5' LTR) followed by transcriptional termination at the normal position.

Progress on Task 4: *Create knock-out construct for the isolated gene. Transfect and isolate ES clones that have undergone gene targeting. Inject ES clones into blastocysts to generate a line of mice that harbor the gene knock-out mutation in their germline. Study phenotype of mice homozygous for the mutation.*

Due to the delay in identifying the *Epi2* gene, we have instead created a novel mutation in the *Nf1* gene (see below). The *NF1* gene encodes neurofibromin, a large protein with multiple isoforms produced as a result of alternative splicing. One of these alternatively spliced exons is exon 23a which encodes 21 amino acids (aa) in the middle of the GAP-related domain. Type I neurofibromin has been defined as not the isoform lacking these additional 21 aa whereas the type II isoform contains them. Others have shown that type II neurofibromin has a 10-fold decrease in GTPase activating protein (GAP) activity, but has a greater affinity for *Ras* (Andersen et al., 1993). In additional, some have suggested that the ratio of type I to type II isoforms may reflect and/or influence differentiation status (Gutmann et al., 1993; Nishi et al., 1991). Therefore, to investigate the function of type II isoform of neurofibromin, we generated mice that specifically lack exon 23a (*Nf123a*$^{-/-}$). So far, using intercrossing of heterozygous mice, we have been able to show that mice homozygous for this mutation are viable and lack type II isoform in brain tissue (Fig. 3). In the past year, we have been characterizing the mutant mice at the histological and behavioral level. We anticipate publication of these results in the coming year.

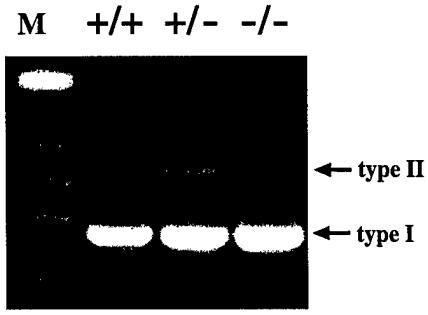

Figure 3. *Nf123a*$^{-/-}$ mice lack the Nf1 type II isoform. RT-PCR analysis of brain RNA using oligonucleotide primers in exons 23 and 24. Type I isoform is 271 bp, type II is 334 bp. M represents the molecular weight marker lane, followed by representative animals of all three genotypes.

Key Research Accomplishments

- Production of a panel of murine acute myeloid leukemia tumors that lack the wild type *Nf1* gene product
- Identification of a locus, *Epi1*, which appears to cooperate with the loss of *Nf1* to cause acute myeloid leukemia (affected in 48% of the tumors in panel)
- Identification of a gene, *Epi2*, which appears to cooperate with the loss of *Nf1* to cause acute myeloid leukemia (affected in 3% of the tumors in panel)
- Identification of a locus, *Epi3*, which appears to cooperate with the loss of *Nf1* to cause acute myeloid leukemia (affected in 3% of the tumors in panel)
- Generation of a novel strain of mouse containing a deletion mutation of the alternatively spliced *Nf1*-exon 23a.

Reportable Outcomes

- An abstract presented at the National Neurofibromatosis Foundation meeting.
- A repository of murine acute myeloid leukemia tumors
- Generation of a strain of mouse lacking the alternatively spliced *Nf1* exon 23a.
- Submission of a grant application to the NIH based on the identification of two genes which appear to cooperate with mutations in the Nf1 gene to cause acute myeloid leukemia
- Successful placement of a former postdoctoral fellow, Tao Yang, in a pathology residency program in Brigham and Women's Hopital in Boston, MA

Conclusions

Our hypothesis has been that progression of juvenile myelomonocytic leukemia (JMML) to acute leukemia (Gadner and Haas, 1992) is the result of genetic mutations in cooperating genes in addition to loss of *NF1*. In the three years of this study, we have successfully identified loci that appear to be candidates for JMML to AML tumor progression. Therefore, this data strongly supports our hypothesis and has provided us with valuable reagents which we can use to experimentally prove our hypothesis.

So what does this research mean to NF1 patients? While most of the tumors associated with neurofibromatosis type 1 (NF1) are benign in nature, unfortunately, malignant transformation of a subset of NF1 tumors can be a serious complication. For example, plexiform neurofibromas are known to transform into malignant nerve sheath tumors (MNPSTs) and JMML is known to progress into acute leukemia (Gadner and Haas, 1992). Because of our research, we now have a handle on the types of genetic changes which lead to the formation, transformation and leukemic progression of NF1-associated JMML. This in turn may lead better prevention, diagnosis, and rational treatment for all malignancies that affect NF1 patients.

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